Exhibit G

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PATENT
Attorney Docket No. 000270-007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

OIPE JC50 DEC 22 1998 PATENT & TRADEMARK OFFICE

In re patent application of )
Steven L. STICE et al )
Serial No. 08/781,752 ) Group Art Unit: 1819
Filed: January 10, 1997 ) Examiner: D. Crouch
For: CLONING USING DONOR NUCLEI FROM DIFFERENTIATED FETAL & ADULT CELLS )

AMENDMENT AND RESPONSE TO OFFICE ACTION AND SUBMISSION OF EXECUTED DECLARATION

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

In response to the non-final Office Action mailed June 22, 1998, and further to the Examiner interview held on December 15, 1998, kindly amend the above-identified application as follows:

IN THE CLAIMS

Kindly cancel Claims 1 through 79, and insert the following new claims:

-80. An improved method of cloning a non-human mammal by nuclear transfer which includes the introduction of a donor cell or nucleus into an enucleated oocyte wherein the improvement comprises using as the donor cell or nucleus a somatic cell or cell committed to a somatic cell lineage capable of division or a nucleus isolated therefrom.

B1

81. An improved method of cloning a non-human mammal by nuclear transfer which includes the introduction of a donor cell or nucleus into an enucleated oocyte wherein the improvement comprises using as the donor cell a somatic cell, cell committed to a somatic cell lineage, or a nucleus isolated therefrom capable of division which has been genetically modified to comprise at least one addition, substitution or deletion modification.

82. A method of cloning a non-human mammal by nuclear transfer comprising the following steps:

(i) inserting a desired non-human somatic cell, cell committed to a somatic cell lineage, or a nucleus isolated therefrom into a non-human mammalian enucleated oocyte under conditions suitable for the formation of a nuclear transfer (NT) unit;

(ii) activating the resultant nuclear transfer unit;

(iii) culturing said activated NT unit until greater than the 2-cell developmental stage; and

(iv) transferring said cultured NT unit to a host non-human mammal such that the NT unit develops into a fetus.

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83. The method of Claim 82, wherein the fetus is further developed into an offspring.

84. The method of Claim 82, wherein a desired DNA is inserted, removed or modified in said somatic cell, cell committed to a somatic cell lineage, or nucleus, thereby resulting in the production of a genetically altered NT unit.

85. The method of Claim 84, which further comprises developing the fetus into an offspring.

86. The method of Claim 80, 81 or 82, wherein the donor cell or nucleus is isolated from mesoderm.

87. The method of Claim 80, 81 or 82, wherein the donor cell or nucleus is isolated from endoderm.

88. The method of Claim 80, 81 or 82, wherein the donor cell is isolated from ectoderm.

B' cont'd

89. The method of Claim 80, 81, or 82, wherein the donor cell or nucleus is a fibroblast cell or cell nucleus.

90. The method of Claim 80, 81 or 82, wherein the donor cell or nucleus is isolated from an ungulate.

91. The method according to Claim 80, 81 or 82, wherein the donor cell or nucleus is isolated from an ungulate selected from the group consisting of bovine, ovine, porcine, equine, caprine and buffalo.

92. The method according to Claim 80, 81 or 82, wherein the donor cell or nucleus is obtained from a non-human mammalian fetus.

93. The method according to Claim 80, 81 or 82, wherein the donor cell or nucleus is an adult non-human mammalian cell or cell nucleus.

94. The method according to Claim 80, 81 or 82, wherein the donor cell or nucleus is selected from the group consisting of epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, muscle cells, and nuclei isolated therefrom.

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95. The method according to Claim 80, 81 or 82, wherein the donor cell or nucleus is obtained from an organ selected from the group consisting of skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organ, bladder, kidney, and urethra.

96. The method of Claim 80, 81 or 82, wherein the oocyte is matured *in vitro* or *in vivo* prior to enucleation.

97. The method according to Claim 82, wherein the oocyte is matured *in vitro* prior to enucleation.

98. The method according to Claim 80, 81 or 82, wherein the oocyte is enucleated by microsurgical methods.

99. The method according to Claim 80, 81 or 82, wherein the oocyte is enucleated about 10 to 40 hours after initiation of *in vitro* maturation.

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100. The method according to Claim 80, 81 or 82, wherein the oocyte is matured *in vivo* prior to enucleation.

101. The method according to Claim 80, 81 or 82, which is used of clone a bovine embryo or offspring.

102. The method of Claim 101, wherein said bovine embryo or offspring is transgenic.--

## REMARKS

Entry of the foregoing amendments, reconsideration and reexamination of the subject application, as amended, pursuant to and consistent with 37 C.F.R. §1.112, and in light of the remarks which follow, are respectfully requested.

At the outset, the Examiner is respectfully thanked for the courteous interview held on December 15, 1998, with James Robl, Ph.D., an inventor of this application, the Examiner and the undersigned. During the interview, the outstanding rejections were discussed in detail. In particular, the Examiner was advised as to Applicants' intent to cancel the product claims and, further, was advised that the claims would be amended consistent with proposed independent claims presented at the interview. It is noted for the record that Applicants have indeed cancelled the product claims herein, and have rewritten the claims consistent with the interview.

Also, the outstanding §112, first paragraph, enablement rejection was discussed in great detail. All of the points raised by the Examiner were respectfully traversed based on the §132 Declaration, which was informally presented to the Examiner at the interview. It was explained that the subject invention comprises a pioneering discovery, i.e., that somatic cells or cells committed to a somatic cell lineage may be used as nuclear transfer donors for cloning desired non-human mammals by nuclear transfer techniques. It was indicated that this was a surprising discovery as it was contravened by previous accepted dogma in the art. Essentially, prior to the present invention, it was thought that once a cell becomes differentiated that it loses its ability to be a suitable donor cell during nuclear transfer. More specifically, as explained in Dr. Robl's Declaration, it was widely thought by researchers working in the area of cloning prior to the present invention that,

once a cell becomes committed to a particular somatic cell lineage, its nucleus irreversibly loses its ability to become "reprogrammed", i.e., to support full-term development when used as a nuclear donor for nuclear transfer.

1. Also, the particular §112 issues raised by the Examiner were discussed. It was explained and substantiated by scientific literature that the efficacy of the subject cloning technique is not dependent upon the particular somatic cell, nor does it depend on the particular *in vitro* maturation technique or the specific culture media used to maintain the nuclear transfer embryo prior to introduction into a female surrogate. Moreover, it was argued, and also substantiated by supporting documentation, that the subject cloning method is generically applicable to non-human mammals. The Examiner was advised that, subsequent to the filing of this application, numerous groups have successfully cloned bovines using fetal and adult cells employing the techniques of the invention and, moreover, sheep, goat, and mice have also been successfully cloned using somatic cells as the donor cell for nuclear transfer. It was also stated and substantiated by supporting documentation that such cloning has been successfully conducted with numerous somatic cell types and using adult and fetal cells. In response to such arguments, the Examiner indicated that she would consider this evidence in detail

when it was formally presented in a subsequent Response and when the Declaration was formally submitted.

Also, as discussed above, the Examiner reviewed proposed independent claims. Based on the Examiner's review, the proposed claims were revised to take into account her suggested changes. Based on the following, it is anticipated that this Response, together with the §132 Declaration and references submitted herewith, should place this application in condition for allowance.

Turning now to the Office Action, Applicants acknowledge the double patenting rejection of Claims 1-34 and 55-77 based on copending U.S. Serial No. 888,283. The Examiner is respectfully requested to hold this rejection is abeyance until this application is otherwise allowable. Moreover, the Examiner is respectfully requested that, if this is the only outstanding issue, to allow this application and to subsequently apply the double patenting rejection, if necessary, in the remaining pending application.

Similarly, Claims 1-34 and 55-77 stand rejected under the doctrine of obviousness-type double patenting as being unpatentable over Claims 1-35 and 47-77 of U.S. Serial No. 888,057. The Examiner is similarly respectfully requested to hold this rejection is abeyance until this application is otherwise allowable and, if this is the only outstanding issue, to allow this application and maintain the double patenting rejection, if necessary, in the remaining pending application.

Claims 1-16, 23, 24, 28-34, 55, 56, 58, 60, 62, 63, 65, 67, 69, 70, 78 and 79 were rejected under 35 U.S.C. §101 on the basis the claims were directed to non-statutory subject matter. This rejection should be moot as the current claims are now restricted to cloning non-human mammals. The basis of the §101 rejection was that the prior claims read on cloning of humans. This obviously was not Applicants' intent and the claims have been amended in order to make this expressly clear. Based on the foregoing, withdrawal of the §101 rejection is respectfully requested.

Claims 33 and 34 were rejected under 35 U.S.C. §112, first paragraph, as assertedly containing subject matter not described in the application so as to enable one skilled in the art to make and/or use the invention. This rejection should also be moot as the current claims no longer contain the phrase "non transformed", which assertedly did not find appropriate support in the disclosure. However, Applicants respectfully maintain that this phrase finds implicit support in the Examples because it is clear that the subject cells which are used as nuclear transfer donors are not transformed. However, in any event, this rejection is now moot as this language does not appear in the current claims.

Claims 1-34 and 55-79 also were rejected under 35 U.S.C. §112, first paragraph, on the basis that the disclosure assertedly only enabled methods for cloning a bovine comprising inserting a fibroblast or the nucleus of the fibroblast isolated from a 45 day pregnancy bovine fetus into the perivitelline space of a bovine oocyte matured *in vitro* to

metaphase II, followed by fusion of the cell or nucleus with the oocyte to form an NT unit with activation being effected by incubating NT units at 26 to 27 hours post-maturation of the oocyte using ionomycin and DMAP under the conditions recited at page 5, first paragraph, of the most recent Office Action, with the resulted nuclear transfer embryo then being cultured using the CR1aa media containing mouse fibroblast feeder cells with the resultant cultured nuclear transfer embryo then being transferred in to a surrogate host bovine for development into a fetus and/or offspring and progeny. Based on the following, the position of the Examiner is respectfully traversed.

It is anticipated that this rejection will be overcome upon consideration of the §132 Declaration by Dr. James Robl which accompanies this Reply. Therein, Applicants address all of the issues raised by the Examiner and provide supporting evidence which substantiates that none of these particular steps are critical to the efficacy of the subject invention. Rather, as discussed above, the present invention involves the generic discovery that cells committed to a somatic cell lineage or somatic cells or nuclei derived therefrom which are capable of division may be used as nuclear transfer donors during nuclear transplantation, and give rise to cloned non-human mammalian embryos, fetuses, and offspring.

Turning to the specific limitations urged to be critical, the Office Action first indicates that the efficacy of the subject invention is dependent upon the age of the donor

somatic cell, and also on the particular somatic cell type. However, this is not correct. To the contrary, as substantiated by subsequent reports, somatic cells of various different ages may be used as successfully as nuclear donors for nuclear transfer. All that is essential is that the somatic cell be capable of division. While the Examiner is correct to the extent that adult cells are less efficient for cloning than are fetal cells, this does not substantiate a position that adult cells cannot be successfully used for cloning. In fact, as disclosed in the subject application, and as has been subsequently reported in the literature, adult cells also can be used for nuclear transfer donors and give rise to viable offspring. For example, as discussed during the recent interview, numerous Japanese groups have recently successfully cloned a number of cows using various different adult cells as the donor cells for nuclear transfer. This is discussed in paragraph 17 of Dr. Robl's Declaration. Moreover, the efficacy of the cloning method is not dependent upon the particular somatic cell type either. As discussed in paragraph 17 of the Robl Declaration, non-fibroblast cells have been successfully used for cloning purposes, e.g., oviduct cells, muscle cells and cumulus cells have been successfully used for cloning non-human mammals.

Instead, all that is important to the invention is that the somatic cells used as the donor be capable of division. As explained in paragraph 18 of the Declaration, cells meeting the appropriate conditions, i.e., which are capable of division, are found in both

fetal and adults. Moreover, while the length of the cell cycle and the life-span of cells shortens with the age of the donor, it is emphasized that cell populations obtained from adult cells comprise cells capable of active division, albeit in lesser numbers than in younger animals. Thus, based on the foregoing, the efficacy of the subject invention does not depend upon the particular age of the donor cell, nor does it depend on a particular somatic type. To reiterate, the only essential feature of the claimed method is that the donor cell be a non-quiescent somatic cell, i.e., a somatic cell capable of division. The importance of division to the efficacy of the invention is discussed, in particular, in the context of fibroblast cells. For example, at page 20 of the subject application, it is reported that fibroblast cells are an ideal cell type because they can be readily obtained from developing fetuses and adult animals in large quantities, and can be easily propagated *in vitro* with a rapid doubling time. Therefore, the importance of active division to the donor cell finds clear support in the disclosure.

The Office Action further alleges that *in vitro* maturation and the particular *in vitro* maturation procedure were critical to the invention. However, this also is not the case. While the Examiner is correct in the fact that *in vitro* matured oocytes are exemplified in the actual working Examples, this is not essential to the efficacy of the subject cloning methods. In fact, cloning has been successfully conducted using *in vivo* matured oocytes. In particular, as discussed in Dr. Robl's Declaration, the inventors recently cloned a

transgenic bovine using *in vivo* matured oocytes. As Dr. Robl explains in his Declaration, *in vitro* matured oocytes were exemplified merely because of supply and cost concerns. They were utilized because immature oocytes (rather than *in vivo* matured oocytes) were available in plentiful numbers, e.g., from slaughterhouse suppliers. However, as substantiated by the fact that a cloned animal has been obtained using *in vivo* matured oocytes, it is clear that the specifics of *in vitro* maturation is not essential to the subject cloning methods.

Nor is the particular means for *in vitro* activation essential to the invention. Again, as properly described by the Examiner, the application exemplifies a particular *in vitro* activation technique which comprises activating the NT unit by incubating 26 to 27 post *in vitro* maturation by incubation in a media comprising specific amounts of ionomycin and DMAP, followed by culturing the NT units in a CR1aa-2mM DMAP medium for four to five hours. However, this particular *in vitro* activation procedure is also not essential to the invention. Indeed, as discussed in detail at pages 14 through 18 of the Declaration by Dr. Robl, those skilled in the art were aware of many different methods for effecting *in vitro* activation at the time the present application was filed.

In general, these methods involve the elevation of intracellular calcium in the egg, which occurs normally *in vivo* upon contacting the egg with sperm. However, this may be effected using many different methods, e.g., using ethanol, electrical shock, cooling,

calcium-free medium, various anesthetics, and a variety of other stimuli. The Declaration summarizes, in paragraph 22, various literature references that teach alternative *in vitro* activation methods, which would be expected to be suitable for use in cloning methods according to the invention. Moreover, for the Examiner's convenience, an overview of different activation procedures is summarized in tabular form at page 17 of the Declaration. Based on the discussion therein, it is clear that there are a number of different activation procedures which can be used for oocyte activation that are conducted by various laboratories around the world, and the use of ionomycin/DMAP was chosen more a matter of convenience than because it was better than other protocols. Thus, contrary to the Office Action, it is not critical to the success of the subject cloning protocols. Therefore, based on the foregoing, withdrawal of this basis of the rejection is also respectfully requested.

Another basis for the §112 enablement rejection was the Examiner's position that the particular culture medium used to maintain the nuclear transfer embryos was essential to the cloning protocol. However, this also is not correct. Again, as substantiated by the Declaration of Dr. Robl, specifically at paragraph 23, the particular exemplified (CR1aa) culture medium is not critical to the efficacy of the invention. In fact, there are many different media that could be used interchangeably for growing bovine embryos in culture. For example, suitable media for culture of bovine embryos include, by way of

example, simple media, complex media, co-culture systems containing cumulus cells, BRL cells, or fibroblast cells, and completely defined media. Moreover, Dr. Robl further advises in his Declaration that, even in his lab, different students are utilizing different media and have had success with the development of embryos. Again, for the Examiner's convenience, a brief overview of different culture media which may be successfully used to maintain bovine embryos in culture is summarized in tabular form at page 17 of the Robl Declaration.

Finally, Applicants respectfully maintain that efficacy of the subject cloning protocol is not limited to bovines. To the contrary, and as discussed at length during the interview, Applicants have made a generic discovery, namely that non-human mammals may be successfully cloned by nuclear transfer using as the nuclear transfer donor a somatic cell or nucleus derived therefrom which is capable of division. The fact that Applicants have made a generic discovery is substantiated by the results of numerous groups who have successfully cloning animals using such cells subsequent to the filing of this application. The fact that the subject cloning protocol is generic to different non-human mammals is substantiated by paragraphs 24 and 25 of Dr. Robl's Declaration. Therein, Dr. Robl advises that somatic cells have been successfully used to clone sheep, mice, bovines (using fetal and adult cells) and goats. Also, the generic nature of the subject discovery is supported by the cross-species nuclear transplantation work also

being conducted by the present inventors. As has been recently well reported in the Press, the present inventors conducted an experiment wherein they produced a nuclear transfer embryo by the insertion of an adult human somatic cell into a enucleated bovine oocyte. After activation, they obtained what appeared to be a blastocyst stage embryo. This further substantiates the fact that adult differentiated cells of different mammalian species can be successfully "reprogrammed" notwithstanding the previous dogma that only very early non-differentiated cells could be used as nuclear transfer donor cells or nuclei.

Therefore, based on the foregoing, it is believed that Applicants have persuasively rebutted all of the bases for the enablement rejection. In particular, Applicants have demonstrated and substantiated, based on the §132 Declaration of Dr. Robl, that the efficacy of the subject invention does not rely upon the age of the donor cell, nor does it depend upon a particular differentiated cell type, or the specific *in vitro* activation procedure or the specific culture medium used to maintain the nuclear transfer embryos. Finally, Applicants have demonstrated that the subject cloning methodology is generic in nature, i.e., that it may be successfully used for cloning different, non-human mammals, including mice, goats, cows, and sheep. As discussed at the interview, the fact that mice have been successfully cloned using somatic cells especially demonstrates the generic nature of the invention given the significant differences in embryonic development between mice and bovines. Also, Applicants respectfully submit that the

present inventors are entitled to broad claims given the pioneering nature of the subject invention. Indeed, as discussed in the Declaration, the subject invention flies in the face of previous dogma in the area of cloning, namely the mistaken idea that only very early non-differentiated cells could be successfully used as donor cells or nuclei for nuclear transfer. Therefore, based on the foregoing, withdrawal of the §112 enablement rejection, which was previously applied to Claims 1-34 and 55-79, is respectfully requested.

Claims 33-34 also were rejected under 35 U.S.C. §112, second paragraph, as assertedly being indefinite in the recitation "transformed". This issue should be moot as this language no longer appears in any of the new claims.

Claims 17-19, 25-27, 71-73, 75 and 77 were rejected under 35 U.S.C. §102(b) as assertedly being anticipated by USP 5,057,420. This rejection is moot as there are no claims now directed to fetuses, offspring, or progeny. As discussed above, these claims have been cancelled without prejudice or disclaimer, and these claims may be subsequently represented in a divisional application.

Claims 20-22, 57, 59, 61, 64, 66, 68, 74 and 76 also were rejected under 35 U.S.C. §102(b) as assertedly being anticipated by Hyttinen (1994), *Bio/Technology*, 12:606-608. This rejection should also be moot as there are no current claims directed to transgenic chimeric and transgenic/chimeric fetuses. Again, these claims were cancelled without prejudice in the interest of expediting allowance of this application.

Claim 29 was rejected under 35 U.S.C. §102(b) as assertedly being anticipated by Sims et al. This rejection is also moot as there are no claims currently directed to a CICM cell line. This subject matter has also been cancelled in order to expedite prosecution.

Claims 17-19, 20-22, 25-27, 29, 57, 59, 61, 64, 66, 68 and 71-77 were also rejected based on Hyttinen and Sims. This rejection is also moot as this subject matter is no longer claimed.

Claims 33, 34, 78 and 79 were rejected under 35 U.S.C. §102(b) as being anticipated by Kono et al. These claims were directed to differentiated cells and human cells made by the claimed process, wherein the cells are not transformed. This rejection is also moot as the claims directed to such differentiated cells and human cells have been cancelled in order to expedite prosecution.

Finally, Claim 31 was rejected under 35 U.S.C. §103(a) as assertedly being unpatentable over Sims et al (1993), *Proced. Natl. Acad. Sci.*, 90:6143-6147, in view of Lovell-Badge et al, *Cold Spring Harbor Symp. Quant. Biol.*, Vol. 50, 707-711. This claim was directed to a transgenic CICM cell line. This rejection is also moot as Applicants no longer claim this cell line. This subject matter has also been cancelled in order to expedite prosecution. The Examiner is also respectfully advised that this subject matter may be resubmitted in a divisional application.

Based on the foregoing, it is believed that the foregoing amendments and remarks, coupled with the §132 Declaration by Dr. Robl and attachments thereto, should place this case in condition for allowance. A Notice to that effect is respectfully solicited. **Moreover, given the great importance of this application, the Examiner is respectfully requested to contact the undersigned after receipt and consideration of this Reply, assuming that any issues remain outstanding. In particular, the Examiner is respectfully requested to contact the undersigned in connection with any proposed Examiner's amendment that would place this case in condition for allowance. Also, Applicants would be amenable to a subsequent telephonic or personal interview with the Examiner, with or without an inventor if necessary, in order to resolve any outstanding issues.**

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: Robin L. Teskin

Robin L. Teskin
Registration No. 35,030

Post Office Box 1404
Alexandria, VA 22313-1404
(703) 836-6620

December 22, 1998

Patent
Attorney's Docket No. 000270-007

# IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| In re Patent Application of | |
| Steven L. STICE et al | Group Art Unit: 1819 |
| Serial No.: 08/781,752 | Examiner: D. Crouch |
| Filed: January 10, 1997 | |
| For: CLONING USING DONOR NUCLEI FROM DIFFERENTIATED FETAL AND ADULT CELLS | |

## DECLARATION OF JAMES M. ROBL Ph.D. PURSUANT TO 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, James M. Robl, Ph.D., declare and state as follows:

(1) I reside at 196 Old Enfield, Belchertown, Massachusetts 01007;

(2) I am a Professor in the Veterinary & Animal Sciences Department at the University of Massachusetts at Amherst Massachusetts;

(3) I have substantial knowledge and expertise in the areas of cloning and transgenic animals. My expertise is substantiated by the attached curriculum vitae.

(4) Based on such expertise, I am frequently asked to give presentations and talks relating to cloning and transgenic animals.

(5) I have reviewed the most recent Office Action issued on June 22, 1998, in the above-identified application. In particular, I have carefully reviewed the enablement rejection that bridges pages 5 to 8 of the Office Action. Based on such review, it is my understanding that the Examiner apparently is of the opinion that the subject application only enables a method for cloning a bovine comprising the specific steps of:

(i) inserting the fibroblast or nucleus isolated from a 45 day bovine fetus into the perivitelline space of a bovine oocyte matured *in vitro* to metaphase II;

(ii) fusing the oocyte and fibroblast or nucleus to form a NT unit; activating the NT unit by incubating such NT unit for 26-27 hours post-maturation in media comprising 5 um ionomycin and 2mM DMAP for 4 minutes;

(iii) culturing the NT units in CR1aa-2mM DMAP media for 4-5 hours;

(iv) and culturing the activated NT units in CR1aa media containing mouse fibroblast feeder cells for 5-8 days after activation and transforming to a host bovine for development into a fetus; as well as offspring and progeny produced by such methods; and producing a bovine CICM cell by the same method except that the transfer to a host bovine is omitted and the cultured activated NT units are

desegregated to produce the inner cell mass of embryos. Based on the following, I respectfully disagree with the Examiner's conclusion.

(6) However, prior to specifically addressing the enablement concerns raised in the office Action, I will summarize some of the novel discoveries which form the basis of the subject invention. It is believed that this discussion will clarify why I am of the opinion that the claims are commensurate in scope with the subject disclosure, especially given the truly pioneering nature of the present invention.

(7) In particular, the novel developments discovered by the inventors of this application include:

i) the successful use of cells committed to a somatic cell lineage for nuclear transfer or transplantation;

ii) the successful use of actively dividing, i.e., <u>non-quiescent</u> cells for nuclear transplantation; and

iii) the use of somatic cell genetic modification to produce genetically modified animals.

Prior to the filing date of this application, to the best of my knowledge, there had been no previous report of the use of cells committed to a somatic cell lineage for successful nuclear transplantation, i.e., that gave rise to viable offspring. I believe this

to be a novel and surprising discovery based on the history of nuclear transplantation prior to the present invention.

(8) In this regard, I note that nuclear transfer first gained acceptance in the 1960's with amphibian nuclear transplantation. (Diberardino, M.A. 1980, "Genetic stability and modulation of metazoan nuclei transplanted into eggs and ooctyes", *Differentiation*, 17-17-30; Diberardino, M.A., N.J. Hoffner and L.D. Etkin, 1984; "Activation of dormant genes in specialized cells", *Science*, 224:946-952; Prather, R.S. and Robl, J. M., 1991, "Cloning by nuclear transfer and splitting in laboratory and domestic animal embryos", In: *Animal Applications of Research in Mammalian Development*, R.A. Pederson, A. McLaren and N. First (ed.), Spring Harbor Laboratory Press.) Nuclear transfer was initially conducted in amphibians in part because of the relatively large size of the amphibian oocyte relative to that of mammals. The results of these experiments indicated to those skilled in the art that the degree of differentiation of the donor nucleus was greatly instrumental, if not determinative, as to whether a recipient oocyte containing such cell or nucleus could effectively reprogram said nucleus and produce a viable embryo. (Diberardino, M.A., N.J. Hoffner and L.D. Etkin, 1984, "Activation of dormant genes in specialized cells.", *Scienc,e* 224:946-952; Prather, R.S. and Robl, J. M.,1991, "Cloning by nuclear transfer and splitting in laboratory and domestic animal embryos", In: *Animal Applications of*

*Research in Mammalian Development*, R.A. Pederson, A. McLaren and N. First (ed.), Spring Harbor Laboratory Press) This work is well documented and was considered dogma prior to the filing of this application.

(9) Much later, in the mid 1980's, after microsurgical techniques had been perfected, researchers, including myself, investigated whether nuclear transfer could be extrapolated to mammals. I worked out the first procedures for cloning cattle (Robl, J. M., R. Prather, F. Barnes, W. Eyestone, D. Northey, B. Gilligan and N.L. First, 1987, "Nuclear transplantation in bovine embryos", *J. Anim. Sci.*, 64:642-647) and my lab was the first to clone a rabbit by nuclear transfer using donor nuclei from earlier embryonic cells (Stice, S.L. and Robl, J. M., 1988, "Nuclear reprogramming in nuclear transplant rabbit embryos", *Biol. Reprod.*, 39:657-664). Also, using similar techniques, bovines (Prather, R.S., FL. Barnes, ML. Sims, Robl, J. M., W.H. Eyestone and N.L. First, 1987, "Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte", *Biol. Reprod.*, 37:859-866) and sheep (Willadsen, S.M., 1986, "Nuclear transplantation in sheep embryos", *Nature*, (Lond) 320:63-65), and putatively porcines (however, this work apparently has never been reproduced) (Prather, R.S., M.M. Sims and N.L. First, 1989, "Nuclear transplantation in pig embryos", *Biol. Reprod.*, 41:414), were cloned by the transplantation of the cell or nucleus of <u>very early embryos</u> into enucleated oocytes.

(10) Moreover, work in our laboratory, and others, investigated the possibility of producing nuclear transfer embryos with donor nuclei obtained from progressively more differentiated cells. However, our results and those obtained by other groups, indicated that when the embryo progresses to the blastocyst stage (the embryonic stage where the first two cell lineages separate) that the efficiency of nuclear transfer decreases dramatically (Collas. P. and J.M. Robl, 1991, "Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos", *Biol. Reprod.*, 45:455-465). For example, it was found that trophectodermal cells (the cells that form the placenta) did not support development of the nuclear fusion to the blastocyst stage. (Collas, P. and J.M. Robl, 1991, "Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos", *Biol. Reprod.*, 45:455-465) By contrast, inner cell mass cells (cells which form both somatic and germ cells) were found to support a low rate of development to the blastocyst stage with some offspring obtained. (Collas P, Barnes FL, "Nuclear transplantation by microinjection of inner cell mass and granulosa cell nuclei", *Mol Reprod Devel.*, 1994, 38:264-267) Moreover, further work suggested that inner cell mass cells which were cultured for a short period of time could support the development to term. (Sims M, First NL, "Production of calves by transfer of nuclei from cultured inner cell mass cells", *Proc Natl Acad Sci*, 1994, 91:6143-6147)

(11) Based on these results, and that of other researchers, it was the overwhelming opinion of those skilled in the art at that time, including myself, that observations made with amphibian nuclear transfer experiments would likely be observed in mammals. That is to say, it was widely thought by researchers working in the area of cloning prior to the present invention that once a cell becomes committed to a particular somatic cell lineage that its nucleus irreversibly loses its ability to become "reprogrammed", i.e., to support full term development when used as a nuclear donor for nuclear transfer. While the exact molecular explanation for the apparent inability of somatic cells to be effectively reprogrammed was unknown, it was hypothesized to be the result of changes in DNA methylation, histone acetylation and factors controlling transitions in chromatin structure that occur during cell differentiation. Moreover, it was believed that these cellular changes could not be reversed.

(12) The discovery made by the present inventors, and subsequently reported by the Roslin Institute after the filing date of this application, i.e., that cells committed to somatic cell lineage could support development when used as nuclear transfer donors, is actually the culmination of a progression of experiments and observations made by our laboratory. For example, we demonstrated in 1990 (unpublished observations, Collas, P. and J.M. Robl, 1991, "Relationship between nuclear

remodeling and development in nuclear transplant rabbit embryos", *Biol. Reprod.*, 45:455-465; Collas P, Barnes FL, "Nuclear transplantation by microinjection of inner cell mass and granulosa cell nuclei", *Mol Reprod Devel*, 1994, 38:264-267) that somatic cells could support development to the blastocyst stage, but not beyond. This seemingly confirmed the general view held by those skilled in the cloning art at that time concerning the irreversible changes to cells that occur during differentiation. In fact, because of this erroneous belief, our first work with somatic cell nuclear transplantation was not conducted with the goal of producing full term offspring. Our laboratory, like others at that time, was of the opinion that this would not be feasible. Rather, we were interested in producing blastocysts by somatic cell nuclear transplantation, and using the resultant blastocyst stage embryos to produce ES-like cells. The thought was that somatic cell-derived ES-like cells might be able to contribute to the development of fully differentiated tissues if grown in association with normal cells in a chimera. This work was successful. (Cibelli, J.B., S.L. Stice, P.J. Golueke, J.J. Kane, J. Jerry, C. Blackwell, F.A. Ponce de Leon and Robl, J.M., 1998, "Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells", *Nature/Biotechnology*, 16: 642-646)

(13) Based on the surprising success of these experiments, we tried to determine whether somatic cell nuclear transplant embryos could support early

development on their own *in vivo* and provide a source of fetal tissue. These experiments were also surprisingly effective and resulted in healthy 40 day fetuses. (Zwada, M.W., J.B. Cibelli, PK. Choi, E.D. Clarkson, P.J. Golueke, S.E. Witta, K.P. Bell, J. Kane, F.A. Ponce de Leon, D.J. Jerry, Robl, J.M., C.R. Freed and S.L. Stice, 1998, "Somatic cell cloning-produced transgenic bovine neurons for transplantation in parkinsonian rats", *Nature Medicine,* 4:569-574)

(14) In the course of these studies we also determined whether these fetal cells could be genetically manipulated *in vitro*, i.e., by the introduction of a heterologous DNA by electroporation, prior to their use as nuclear transplant donors. These experiments were effected because an important goal of cloning, if not the most important goal of cloning, is to provide a reproducible source of cells having a desired genotype, e.g., which express a particular transgene. Thereby, the resultant cloned embryos or animals can be used to produce a desired gene product or for cellular transplantation therapies. However, it was by no means predictable that these experiments would be successful. To the contrary, I am of the opinion, based on the state of the art at the time of the invention, that the prevailing expectation would have been that these cells, given their differentiated state, coupled with the fact that they were manipulated in tissue culture and then transfected with a foreign DNA, would either not give rise to nuclear transfer embryos at all, or would produce embryos that

would only differentiate to early stages. However, as can be seen from the experiments, the results of which are reported in this application, quite surprisingly, these cells when inserted in enucleated bovine oocytes gave rise to apparently perfectly healthy 40 day transgenic bovine embryos.

(15) Thereupon, based on the surprising success of the above-described experiments, we then attempted to determine whether somatic cell nuclear transplant embryos would give rise to viable full-term bovine offspring, and more desirably, transgenic viable full-term bovine offspring. As the Examiner is aware, and has been well reported in the press, it was astoundingly discovered that cells which are committed to a differentiated cell type, which cells were moreover made transgenic (transgenic fetal fibroblasts) when used as nuclear transfer donors, gave rise to healthy, transgenic bovine offspring. Moreover, these results have been successfully repeated by us and other groups. In fact, based on the reproducibility and efficiency of the subject cloning technique, the licensee of this application has entered into a collaboration with Genzyme Transgenics Corporation to make cloned transgenic bovines that produce a polypeptide (HSA) in their milk. It is further noted that using the basic cloning methods which are the subject of this application, a transgenic bovine that contains the HSA gene has been successfully obtained. With this basic understanding of the state of the nuclear transfer art that existed prior to the present

invention, and the developments and discoveries that culminated in the subject invention, I will now address the various enablement concerns raised by the Examiner.

(16) Types and Age of Differentiated Cells Used for Nuclear Transfer

As discussed, our invention involves the generic discovery that cells committed to a somatic cell lineage, which optionally are transgenic, can be used as nuclear transfer donors to produce viable fetuses and offspring. Contrary to the Examiner's position, the efficacy of the invention does not require that such somatic cells be fibroblasts isolated from 45 day old bovine offspring. In fact, our results and those of others supports our claim that a wide variety of types and ages of cells committed to a somatic cell lineage can be used successfully for nuclear transfer. Moreover, our results and those of others further support the view that these cells may be used to produce cloned transgenic animals.

(17) For example, two calves have been produced at the Ishikawa Prefecture Livestock Research Centre in Japan from oviduct cells collected from a cow at slaughter. (Hadfield, P. and A. Coghlan, "Permature birth repeats the Dolly mixture", *New Scientist*, July 11, 1998) Also, Jean-Paul Renard from INRA in France has produced a calf from muscle cells from a fetus. (MacKenzie, D. and P. Cohen, 1998., "A French calf answers some of the questions about cloning", *New Scientist*, March 21) Further, David Wells from New Zealand has produced a calf from fibroblast cells

from an adult cow. (Wells, D.N., 1998, "Cloning symposium: Reprogramming Cell Fate - Transgenesis and Cloning", Monash Medical Center, Melbourne, Australia, April 15-16)

(18) Moreover, our experiments to date indicate that cells obtained from fetuses, calves, young adults and aged adults all can be grown in culture and can be used as nuclear donor cells to produce cloned animals. However, it should be noted that the length of the cell cycle and the life-span of the cells shortens with the age of the donor. Furthermore, the percentage of healthy embryos produced from adult cells decreases with the age of the animal. This suggests that actively dividing cells can more readily support development following nuclear transfer than cells progressing towards a quiescent state. However, it does <u>not</u> support a proposition that <u>only</u> cells of a certain age can be used as suitable nuclear transfer donors.

(19) Interestingly, there is considerable variation in the length of the cell cycle within these populations. In fetal populations, some cells divide as rapidly as every 12 hours with most having divided by 24 hours. By contrast, in cell populations obtained from adult cells, a few cells will divide relatively quickly, but most require more than 24 hours to divide. These results explain why adults can be cloned, but the efficiency appears to be much lower than with fetuses. These observations also lend credence to the observation made by us, namely that actively dividing cells are capable

of being used as donor nuclei, or cells, during nuclear transfer to produce viable embryos.

This is contrary to the work of the Roslin Institute (published after the filing date of this application), which instead reported the use of quiescent, i.e., non-actively dividing cells, to produce cloned sheep. (Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS, "Viable offspring derived from fetal and adult mammalian cells", *Nature*, 1997, 1385:810-813) In fact, this significant difference in our cloning method *vis-a-vis* that reported by the Roslin Institute was discussed by us in a letter to the Editors of *Science*, 281:1611 (1998) (a copy of which is attached to this Declaration). In my opinion, this difference explains why the cloning methods reported in this application are highly reproducible.

(20) Non-Criticality of *In vitro* Maturation

The Examiner has seemingly concluded that the use of *in vitro* matured oocytes is critical to the efficacy of the invention. This, however, is not correct. While the Examiner is correct in the fact that the use of *in vitro* matured oocytes is exemplified in the actual working examples, this is not essential to the efficacy of the subject cloning methods. *In vitro* matured oocytes were used largely because of supply and cost concerns. Essentially, immature oocytes, rather than *in vivo* matured oocytes were used because we did not have access to *in vivo* matured oocytes and because *in vitro*

mature oocytes can be obtained in plentiful numbers (e.g., from slaughterhouse suppliers). However, as stated in our patent application, *in vivo* matured oocytes can also be used for nuclear transfer. For example, *in vivo* matured oocytes can be collected from either non-superovulated or ovulated cows or heifers and used for enucleation. In fact, *in vivo* oocytes have recently been used by us for cloning and a calf was successfully produced this month. Therefore, it is clear that the efficacy of the subject cloning method does not rely on a particular *in vitro* matured procedure, or even that *in vitro* matured oocytes be used at all.

(21) Oocyte Activation Protocol

Also, contrary to the Office Action, the efficacy of the subject cloning methods is not limited to the specific activation conditions exemplified in the working examples. As explained above, the truly novel discovery made by us are that (1) cells committed to a somatic cell lineage can support development to term following nuclear transfer; (2) the use of actively dividing, non-quiescent, cells for nuclear transplantation, and (3) the use of somatic cell genetic modification to produce genetically modified animals. The specific activation protocol that is used was not critical to the cloning method.

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(22) With respect thereto, my laboratory has studied the activation process in great detail over the past twelve years. Activation is a process that involves the

elevation of intracellular calcium in the egg. The sperm normally produces oscillations in calcium concentration that last for several hours. Artificial activation protocols have been used on eggs for many years. Early work indicated that ethanol, electrical shock, cooling, calcium-free media, various anesthetics and a variety of other stimuli could cause activation. (Whittingham, D.G., 1980, "Parthenogenesis in mammals", *Oxford Rev. Reprod, Biol.*, 2:205-231) In more recent years, with the development of procedures for measuring intracellular calcium and the various intracellular responses to calcium, more specific approaches have been developed. For example, we now know that electrical pulses cause transient increases in intracellular calcium by inducing pores in the membrane and allowing calcium to flood into the cell from the extracellular media. (Fissore, R.A. and Robl, J.M., 1992, "Intracellular calcium response of rabbit oocytes to electrical stimulation", *Mol. Reprod. Devel.*, 32:9-16; Collas, P., J.J. Balise, G.A. Hofman and Robl, J.M., 1989, "Electrical activation of mouse oocytes", *Theriogenology*, 32:835-844; Robl, J.M., P. Collas, R. Fissore and JR. Dobrinsky, 1992, "Electrically induced fusion and activation in nuclear transplant embryos", In: *Guide to Electroporation and Electrofusion*; D. Chang, B.M. Chassy, J.A. Saunders and A.E. Sowers (ed.), Academic Press, Inc., San Diego, CA; Collas, P., R. Fissore, J. M. Robl, E.J. Sullivan and F.L. Barnes, 1993, "Electrically-induced calcium elevation, activation and parthenogenetic development of bovine oocytes", *Mol. Reprod. Devel.*,

34:212-223; Collas, P., R. Fissore and J.M. Robl, 1993, "Preparation of nuclear transplant embryos by electroporation", *Anal. Biochem.*, 208:1-9) Multiple pulses can be used to duplicate sperm-induced calcium oscillations. Injection of such intracellular second messengers such as IP3, or its long acting analogues, GTP, or its long acting analogues, or calcium itself can duplicate sperm-induced calcium rises. Other compounds that cause calcium rises, although less physiological, are ethanol and calcium ionophores. (Fissore, R.A. and Robl, J.M., 1993, "Sperm, inositol trisphosphate and thimerosal induced intracellular Ca2+ elevations in rabbit eggs", *Devel. Biol.*, 159:122-130; Fissore, R.A. and Robl, J.M., 1994, "Mechanism of calcium oscillations in fertilized rabbits eggs", *Devel. Biol.*, 166:634-642; Fissore, R.A., Pinto-Correia, C. and J.M. Robl, 1995, "Inositol trisphosphate-induced calcium release in the generation of calcium oscillations in bovine eggs", *Biol Reprod.*, 53:766-774; Collas, P., Chang, T., Long, C. and J.M. Robl, 1995, "Inactivation of histone H1 kinase by $Ca^{2+}$ in rabbit oocytes", *Mol. Reprod. Devel.*, 40:253-258.) The second part of the activation event is a decrease in a cell cycle regulatory kinase called MPF. This results in a decrease in the phosphorylation of many different proteins in the cell and the progression to interphase. This part of the process can be duplicated by various kinase inhibitors. MPF can be inactivated directly by inhibiting protein synthesis and compounds like puromycin and cycloheximide have

been used successfully in oocyte activation protocols. Currently, there are a number of different combinations of the above that are being used successfully for oocyte activation in various laboratories around the world. Our use of the ionomycin/DMAP procedure was more a matter of convenience than a view that it was better than various other protocols, and it is certainly not critical for the success of the procedure.

For the Examiner's convenience, a brief overview of different activation procedures known prior to the filing of this application are summarized below:

| Cell type | Activation | Culture media | Reference |
|---|---|---|---|
| Blastomeres | Electrical | B2 + oviductal cells | (Ectors et al. 1995) |
| Blastomeres | Electrical | CRI | (Zakhartchenko et al. 1995) |
| Blastomeres | Electrical | Bovine oviduct epithelial cells | (Campbell et al. 1993) |
| Blastomeres | Electrical | Modified Brinsters Ovum Culture Medium | (Bames et al. 1993) |
| Blastomeres | Chilling | Sheep oviduct | (Westhusin et al. 1996) |
| ICM cells | Chilling | CRI + MEM + BME | (Keefer et al. 1994) |
| Oogonia | Ion+DMAP | TCM 199 + Steer serum | (Lavoir et al. 1997) |
| Blatomeres | Electrical | TCM 199 + calf serum | (Takano H. 1996) |

Bames, F. L., Collas, P., Powell, R., King, W. A., Westhusin, M., and Shepherd, D. (1993). "Influence of Recipient Oocyte Cell Cycle Stage on DNA Synthesis, Nuclear Envelope Breakdown, Chromosome Constitution, and Development in Nuclear Transplant Bovine Embryos." *Molecular Reproduction and Development*(36), 33-41.

Campbell, K. H. S., Ritchie, W. A., and Wilmut, I. (1993). "Nuclear-Cytoplasmic Interactions during the First Cell Cycle of Nuclear Transfer Reconstructed Bovine Embryos: Implications for Deoxyribonucleic Acid Replication and Development." *Biology of Reproduction*(49), 933-942.

Ectors, F.J., Delval, A., Smith, L. C., Touati, K., Remy, B., Beckers, J.-F., and Ectors, F. (1995). "Viability of Cloned Bovine Embryos After One or Two Cycles of Nuclear Transfer and In Vitro Culture." *Theriogenology*(44), 925-933.

Keefer, C. L., Stice, S. L., and Matthews, D. L. (I 994). "Bovine Inner Cell Mass Cells as Donor Nuclei in the Production of Nuclear Transfer Embryos and Calves." *Biology of Reproduction*(50), 935-939.

Lavoir, M.-C., Rumph, N., Moens, A., King, W. A., Plante, Y., Johnson, W. H., Ding, J., and Betteridge, K. J. (I 997). "Development of Bovine Nuclear Transfer Embryos Made with Oogonia." *Biology of Reproduction*(56), 194-199.

Takano H., K. C., Kato Y, Tsunoda Y. (I 996). "Cloning of bovine embryos by multiple nuclear transfer." *Theriogenology*, 47, 1365-1373.

Westhusin, M. E., Collas, P., Marek, D., Sullivan, E., Stepp, P., Pryor, J., and Bames, F. (1996). "Reducing the Amount of Cytoplasm Available for Early Embryonic Development Decreases the Quality But Not Quantity of Embryos Produced by In Vitro Fertilization and Nuclear Transplantation." *Theriogenology*(46), 243-252.

Zakhartchenko, V., Wolf, E., Palma, G. A., and Brem, G. (I 995). "Effect of Donor Embryo Cell Number and Cell Size on the Efficiency of Bovine Embyro Cloning." *Molecular Reproduction and Development*(42), 53-57.

(23) <u>Culture Medium Used to Maintain Nuclear Transfer Embryos</u>

Also, contrary to the Office Action, a particular culture medium is <u>not</u> critical to the efficacy of the invention. Indeed, there are many different media that can be used interchangeably for growing bovine embryos in culture. The only important factor with

respect to such culture media is that they be based on a Krebbs-Ringer bicarbonate solution. This solution has a variety of salts and a bicarbonate buffer system that is based on the analysis of blood serum components. Such media generally contain an energy source, antibiotics and usually some complex component such as serum or co-cultured cells. In use currently for culture of bovine embryos are simple media, complex media, co-culture systems with cumulus cells, BRL cells or fibroblast cells, and completely defined media. (Different method in current usage can also be found in the Table in paragraph (22) supra.) Moreover, in my laboratory, one student is using a complex media with a cumulus cell co-culture while another student is using a simple media with a fibroblast co-culture. Both students are doing nuclear transplantation work and having success with development of embryos. The reason for using the different culture systems in the same lab simply is that one student is more comfortable with one system, and the other is more comfortable with the other system. Therefore, it is apparent that a specific culture media is not essential to the efficacy of the subject invention.

(24) Use of Method for Produce Other Cloned Species (Non-Bovines)

In my expert opinion, the subject cloning methods can be used to clone different mammals, i.e., other than bovines. That is to say, I am of the opinion that the basic discovery made by us that cells committed to a somatic lineage, preferably non-

quiescent cells, can be used as nuclear transfer donors to produce nuclear transfer embryos that give rise to viable fetuses and offspring, can be extrapolated to different, i.e., non-bovine mammals. In fact, subsequent to the filing of this patent application, I am aware that differentiated cells have reportedly been successfully used to produce cloned sheep and mice. (Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS, "Viable offspring derived from fetal and adult mammalian cells", *Nature*, 1997, 1385:810-813; Wakayama T, Perry ACF, Zucconi M, Johnsoal KR, Yanagimachi R., "Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei", *Nature*, 1998, 394:369-374)

(25) The generic nature of our discovery is further supported by recently reported experiments conducted by our lab relating to the efficacy of cross-species nuclear transplantation. Specifically, we conducted an experiment wherein we successfully produced a nuclear transfer fusion embryo by the insertion of an adult differentiated cell (obtained from the cheek of an adult human donor) into an enucleated bovine oocyte. This further substantiates the fact that adult differentiated cells of different species can be successfully "reprogrammed", notwithstanding the previous dogma that existed prior to the present invention, i.e., the widely-held but mistaken belief that only very early non-differentiated cells could be used for nuclear transfer donor cells or nuclei.

(26) In summation, I hope that I have adequately addressed all of the Examiner's enablement concerns. For the reasons set forth above, and those enumerated in the present application, I am of the opinion that the efficacy of the subject invention does not depend upon the specific parameters, e.g., cell type, age, *in vitro* maturation, oocyte activation method, utilized in our working examples. Moreover, I believe that the Patent Office's conclusion is further untenable given the truly pioneering nature of the invention and further based on the fact that subsequent to the invention, differentiated cells have reportedly been successfully used by numerous groups to produce nuclear transfer embryos and cloned offspring.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 12/9/98 James M. Robl

James M. Robl, Ph.D.

# CURRICULUM VITAE


**Name:** *James M. Robl*

**Address:** Paige Laboratory
University of Massachusetts
Amherst, Massachusetts 01003
Phone: (413) 545-5565
FAX: (413) 545-6326
E-mail: robl@vasci.umass.edu




**Education:**

B.S. (1977) Kansas State University, Manhattan, Kansas
M.S. (1979) Kansas State University, Manhattan, Kansas
Ph.D. (1983) University of Illinois, Urbana, Illinois
Post-Ph.D. (1983-1985) University of Wisconsin, Madison

**Positions:**

Research Assistant, Department of Animal Science, Kansas State University, Manhattan, Kansas, 1977-1979.
Research Assistant, Department of Animal Science, University of Illinois, Urbana, Illinois, 1979-1980 and 1981-1982.
NIH Fellow, Reproductive Biology Training Grant, University of Illinois, Urbana, Illinois, 1980-1981 and 1982-1983.
Research Associate, Department of Animal Science, University of Wisconsin, Madison, Wisconsin, 1983-1985.
Assistant Professor, Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts, 1985-1991.
Associate Professor, Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts, 1991-1997.
Professor, Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts, 1997-present.

**Patents:**

Prather, R.S., F. Barnes, J. Robl, N.L. First and V. Simmon. 1991. Multiplying bovine embryos. No. 4,994,384.

Inventor on 11 additional patent filings

**Books:**

Monastersky, G.M. and J.M. Robl. 1995. Strategies in Transgenic Animal Science. American Society for Microbiology Press, Washington, D.C.

**Documentaries:**

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